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Genetic characterization of *Artemia tibetiana* (Crustacea: Anostraca)

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The brine shrimp *Artemia* consists of a number of bisexual species and a large number of parthenogenetic forms, which collectively, inhabit a wide range of hypersaline habitats. A recently described species (*A. tibetiana*) from a carbonate lake (Lagkor Co) in Tibet at an altitude of 4490 m has been tested with New World (*A. franciscana* USA, and *A. franciscana* feral population Vietnam) and Old World species (*A. salina*, *A. urmiana*, *A. sinica*) for cross fertility. These tests show complete infertility between *A. tibetiana* and *A. franciscana*. Between *A. tibetiana* and *A. urmiana*, *A. sinica* partial fertility through to F_2 and F_3 generations is evident. Allozyme and RAPD comparison of *A. tibetiana* with *A. franciscana* (USA), *A. franciscana* (Vietnam), *A. sinica* (Mongolia) and *A. urmiana* (Iran) show that *A. tibetiana* is similar to other bisexual species in mean heterozygosity (0.074) but has a somewhat higher proportion of polymorphic loci (40%, similar to that of *A. urmiana*). The genetic distance between *A. tibetiana* and *A. franciscana* is 0.730, between *A. tibetiana* and *A. urmiana* is 0.475 and that between *A. tibetiana* and *A. sinica* is 0.114. F_{IS} estimates for *A. tibetiana* differ significantly from zero for six loci, mainly because of lack of fit to Hardy–Weinberg expectations. This may suggest that even within the limited area of Lagkor Co there are genetically distinct populations. *Biological Journal of the Linnean Society*, 2002, **75**, 333–344.

ADDITIONAL KEYWORDS: allozymes – *Artemia* – brine shrimp – genetic divergence – RAPDs – reproductive isolation – speciation.

INTRODUCTION

The brine shrimp *Artemia* (Crustacea, Anostraca) inhabits both inland and coastal saline and hypersaline lakes. The genus is a complex of species and superspecies defined largely, though not completely, by the criterion of reproductive isolation (Browne & Bowen, 1991; Pilla & Beardmore, 1994).

Artemia bisexual species are grouped in the New World species, i.e. *Artemia franciscana* Kellogg (1906) and *A. persimilis* Piccinelli & Prosdocimi (1968), and Old World species, i.e. *A. salina* Leach (1819) (in Triantaphyllidis *et al.*, 1997), *A. urmiana* (Günther, 1890), *A. sinica* (Cai, 1989), *Artemia* sp. from Kazakhstan (Pilla & Beardmore, 1994) and *A. tibetiana* (Abatzopoulos, Zhang & Sorgeloos, 1998). The parthenogenetic forms are grouped, controversially and not very logically, under the binomen *A. parthenogenetica* (Barigozzi, 1974). All bisexual species are diploid and have $2n = 42$ except for *A. persimilis* which has $2n = 44$. A great variety of ploidies has been observed in parthenogens (Barigozzi, 1974; Abatzopoulos *et al.*, 1986; Triantaphyllidis *et al.*,

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1996), while in all *Artemia* species the phenomenon of aneuploidy is a common feature especially in the first larval stages.

The characterization of *Artemia* species and/or populations has been a continuous endeavour during the second half of the previous century. Recently, new multidisciplinary approaches have been used to define *Artemia* species (for an extensive review see Triantaphyllidis *et al.*, 1997).

The brine shrimp *Artemia* has a diverse geographical distribution. Its environments vary considerably in terms of water anionic composition, climatic conditions and altitude (Triantaphyllidis *et al.*, 1998 and references therein). Depending on the prevailing anions, *Artemia* may inhabit chloride, sulphate or carbonate waters and/or combinations of two or even three major anions (Bowen *et al.*, 1985; Bowen *et al.*, 1988). The organism can be found in climatological conditions ranging from humid-subhumid to arid (Vanhaecke *et al.*, 1987) and in altitudes from sea level up to 4500 m (Xin *et al.*, 1994; Triantaphyllidis *et al.*, 1998). The *Artemia* population found in Lagkor Co, Tibet, P.R. China belongs to the latter.

Lagkor Co is a carbonate lake situated 4490 m above sea level in the arid temperate plateau zone of Tibet at 84°13′E and 32°03′N. This alkaline lake (pH 8.8) has a salinity of 55–65‰ and the temperature varies from a maximum of about +24 °C to a minimum of about −26 °C with an average annual air temperature of about 1.6 °C (for more information on the lake's characteristics and hydrochemical type see Mianping, 1997; Zunying *et al.*, 1998).

Dong *et al.* (1982) (cited in Mianping, 1997) characterized the *Artemia* from Lagkor Co as a cryophilic *A. salina*. Abatzopoulos *et al.* (1998), using mainly biometrics of cysts and nauplii, cytogenetics and cross fertility tests with known *Artemia* species, found enough evidence to support the view that *Artemia* from Lakgor Co is a new species and proposed the name *Artemia tibetiana*. Zunying *et al.* (1998) compared biological features (such as cyst diameter and length of nauplii) between *Artemia* from Lagkor Co and 13 other populations within China and elsewhere, and concluded that the Tibetan population belongs to the larger type of *Artemia*. Using AFLP markers, Sun *et al.* (1999) analysed 15 *Artemia* populations/species and found that *A. tibetiana* seems to be differentiated from all other bisexual species, including *A. sinica*, thus supporting the idea that it is a new species.

In this paper, we present for the first time a detailed description of this new *Artemia* species. We tried to determine reproductive isolation patterns through cross-breeding laboratory tests. In addition, allozyme electrophoresis and RAPD analyses were used to investigate the genetic differentiation between *A. tibetiana* and other bisexual *Artemia* species from the New and Old World.

MATERIAL AND METHODS

Populations investigated

The origins of the *Artemia* populations studied and the abbreviations used are given in Table 1.

Culture conditions

The hatched nauplii were transferred to 1-litre cylindroconical glass tubes containing 0.25 µm-filtered Instant Ocean artificial seawater. The initial number of nauplii per tube was 200, the salinity 50‰ and the temperature 23 ± 1 °C. For *A. tibetiana* the culture temperature was kept below 20 °C, which was found to be optimal for this psychrophilic species. For more details on culturing procedures and the feeding schedule see Triantaphyllidis *et al.* (1997).

Table 1. List of bisexual populations, their *Artemia* Reference Center (ARC) cyst bank code number, their species designation (if known) and abbreviations used

Population	ARC cyst bank code *number*	Species designation	Abbreviation
San Francisco Bay, California, USA	1258	*A. franciscana*	FRA
Vinh Chau, Vietnam	1084	*A. franciscana*	VC
Yuncheng, Shanxi Province, P.R. China	1218	*A. sinica*	SIN
Yimeng, Inner Mongolia, P.R. China	1188	*A. sinica*	YIM
Namibia	1186	Parthenogenetic	NAM
Aibi Lake, Xinjiang Province, P.R. China	1236	Parthenogenetic	AIB
Sfax, Tunisia	1268	*A. salina*	SAL
Argentina	1321	*A. persimilis*	PER
Urmia Lake, Iran	1229	*A. urmiana*	URM
Lagkor Co, Tibet, P.R. China	1347	*A. tibetiana*	TIB

BREEDING TESTS

Reproductive compatibility was evaluated by single-pair reciprocal crosses of adults between *A. tibetiana* (TIB) and four other bisexual species, i.e. *A. franciscana* (FRA), *A. salina* (SAL), *A. urmiana* (URM), *A. sinica* (SIN) and *A. sinica* from Yimeng, Inner Mongolia (YIM) (see Table 2). Virgin females were isolated from the stock cultures well before they reached sexual maturity and kept for at least 14 days to ensure non-impregnation; according to Bowen (1962) female *Artemia* do not store sperm. Males were collected directly from stock cultures. Each pair was placed in a 50-mL Falcon tube (salinity 55–60‰). Crosses were inferred to be fertile when full/intact cysts or live nauplii were produced. The viability of the cysts was determined according to their ability to hatch (and give live nauplii) in standard conditions following deactivation of diapause (i.e. dehydration at 38 ± 1 °C for at least 48 h, rehydration/dehydration cycles and/or hibernation at −30 °C for at least 2 weeks). The viability of the nauplii was tested by raising them to sexually mature adults.

Table 2. Intraspecific and interspecific crosses between bisexual *Artemia* from Old and New World

		Type of cross (fertile/total)		
Female/Male	Parental	F_1	F_2	F_3
FRA/FRA (c)	12/12	10/10 (8)	8/8 (6)	NT
SAL/SAL (c)	9/10	6/7 (5)	7/8 (3)	NT
URM/URM (c)	10/12	5/6 (4)	4/4 (4)	NT
SIN/SIN (c)	9/10	5/7 (5)	8/9 (4)	NT
YIM/YIM (c)	11/12	8/11 (6)	3/4 (2)	NT
TIB/TIB (c)	6/7	10/12 (4)	7/9 (5)	NT
FRA/TIB	0/9	–	–	–
TIB/FRA	0/8	–	–	–
SAL/TIB	0/10	–	–	–
TIB/SAL	0/7	–	–	–
URM/TIB	3/10	2/6 (2)	0/3 (2)	–
TIB/URM	2/4	2/6 (2)	0/2 (1)	–
SIN/TIB	4/11	0/2 (1)	–	–
TIB/SIN	3/4	4/6 (3)	2/5 (2)	0/4 (1)
YIM/TIB	5/10	12/20 (5)	5/14 (3)	2/13 (3)
TIB/YIM	7/11	11/19 (6)	7/15 (5)	1/9 (3)

Numbers in parentheses indicate the number of fertile crosses of the previous generation from which pairs were drawn. NT, not tested; (c), control; FRA, *A. franciscana*; SAL, *A. salina*; URM, *A. urmiana*; SIN, *A. sinica*; YIM, *A. sinica* from Yimeng (Inner Mongolia); TIB, *A. tibetiana*.

ALLOZYME ASSAYS

Adult individuals from the populations FRA (*A. franciscana*), VC (*A. franciscana*, originated after the inoculation of this species in Vinh Chau, Vietnam in 1982), TIB (*A. tibetiana*), YIM (*A. sinica*) and URM (*A. urmiana*) were isolated from stock cultures and prepared for allozyme analysis following the procedures described by Abreu-Grobois & Beardmore (1982). Approximately equal numbers of randomly drawn adult males and females (48–77 from each population) were used for allozyme analyses. Allozyme variation was assayed by standard horizontal starch (Connaught, 10%) gel electrophoresis. A total of 13 enzymes encoded by 20 loci were chosen on the basis of the work of Abreu-Grobois (1983). All enzymes and buffer systems are shown in Table 3. The protocols followed were those described by Harris & Hopkinson (1976) and Murphy *et al.* (1990). Staining of gels followed the methods of Abreu-Grobois (1983) and Pilla (1992). Allelic variants were designated by numeric values relative to the commonest band in FRA, which was arbitrarily assigned an electrophoretic mobility of 100.

Calculation of genetic variability parameters (allele and genotype frequencies, *F* statistics, genetic distances) used various programs from two statistical packages: BIOSYS-1, Release 1.7 (Swofford & Selander, 1981) and TFPGA, ver. 1.3 (Miller, 1997). Exact tests (with no pooling) were used to determine Hardy–Weinberg (H-W) deviations and allele frequency heterogeneity. The sequential Bonferroni tech-

Table 3. Buffer systems, enzymes and Enzyme Commission (E.C.) numbers

Buffer	Enzyme	E.C. number
TEC_a	Esterase & Esterase D	3.1.1.–
	Phosphogluconate dehydrogenase	1.1.1.44
	Malate dehydrogenase	1.1.1.37
	Lactate dehydrogenase	1.1.1.27
	Isocitrate dehydrogenase	1.1.1.42
TEB_b	Glutamate-oxaloacetate transaminase	2.6.1.1
	Leucine aminopeptidase	3.4.11.1
	Phosphoglucomutase	5.4.2.2
$POULIK_c$	Glucosephosphate isomerase	5.3.1.9
	Peptidase	3.4.11.–
	NADP-malate dehydrogenase	1.1.1.40
	Superoxide dismutase	1.15.1.1
	Amylase	3.2.1.1

a, Tris–EDTA–Citrate; *b*, Tris–EDTA–Borate; *c*, Tris citrate/borate hydroxide; –, variable subunit number.

nique (Rice, 1989; Sokal & Rohlf, 1995) was employed in all cases where multiple tests were performed. The population-specific test level was preferred (whenever possible) to control the type-I error rate.

Extraction and analysis of DNA

Cysts (100 mg) were suspended in 1 mL Sodium EDTA buffer (750 mM NaCl; 250 mM Na_2EDTA) to which 10 μL Tris buffer was added (Tris stock 1 M at pH 8). The samples were homogenized at 4 °C with a potter (Braun, Germany) at 1000 r.p.m. for 1 min. Then 20 μL of proteinase K (stock 10 mg/mL) and 50 μL SDS (stock 10%) were added to 500 μL of homogenate. After 30 min incubation at 65 °C, the sample was extracted once with 500 μL phenol-chloroform (1:1) and once with 500 μL of chloroform. To the supernatant (12000*g*, 5 min), 650 μL of water and 1300 μL of ice-cold isopropanol were added. The precipitated DNA (1 h at −20 °C) was pelleted at 12000*g* for 15 min and resuspended in distilled water. The RNA in the samples was degraded by incubation in the presence of RNAse A (0.2 mg/mL) at 37 °C for 15 min. The DNA samples were stored at −80 °C. The DNA concentration was measured with a spectrofluorophotometer (Shimadzu RF-1501). Therefore aliquots of the sample were dissolved in water containing ethidium bromide (final concentration 0.3 μg/mL). DNA was measured by fluorometry (excitation and emission wavelengths at 325 and 563 nm, respectively). At those light wavelength settings (as verified experimentally), residual proteins in the samples do not contribute to the emission of the light. The readings were converted to DNA concentrations with the help of a herring sperm DNA (Roche Molecular Biochemical, Brussels, Belgium) standard series (0–333 pg/μL). The DNA in the sample was diluted to 50 ng/μL.

RAPD reactions were performed with two primers, namely ERIC1R (5′ATGTAAGCTCCTGGGGA TTCAC3′) and ERIC1RA (5′ATGTAAGCTCCTGG GGATTCAG3′) (Eurogentec, Seraing, Belgium) using one primer at a time. The PCR (Hybaid PCR express, Labsystems, Brussels, Belgium) conditions were as follows: 94 °C for 2 min; 5 × 94 °C for 30 s, 40 °C for 2 min, 68 °C for 8 min; 25 × 94 °C for 30 s, 65 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 5 min. Therefore 100 ng total DNA and 1 U DNA polymerase mixture (Expand High Fidelity PCR system, Roch Molecular Biochemicals) were mixed into 10 mM TrisHCl, 50 mM KCl, 5 mM $MgCl_2$ containing 20 μM primer and 0.2 mM dNTP's (final reaction volume, 50 μL).

The generated DNA fragments were separated on an ExcelGel system (horizontal polyacrylamide gel electrophoresis) and silver stained (Pharmacia). A 100-bp ladder (Promega) was loaded as a reference. The gel was dried and scanned by a flatbed scanner (Scanjet II cx, HP) at 400 dpi. The image was processed with the Gelcompar software (Applied Maths, Kortrijk, Belgium). Pearson correlation coefficients between the profiles were calculated which served as input for a UPGMA (unweighted pair-group method with arithmetic averages) dendrogram.

RESULTS

Laboratory reproductive isolation

Population and/or species abbreviations and data from reproductive isolation tests are listed in Table 2. The data reveal that complete infertility exists between *A. tibetiana* and the New World bisexual species *A. franciscana*. The same is true of crosses between *A. tibetiana* and the Western Old World bisexual *A. salina*. This must be the result of substantial post-mating isolating barriers at the laboratory level, since pairing was always observed. When matings between *A. tibetiana* and Eastern Old World bisexual species (i.e. *A. urmiana* and *A. sinica*) are considered, the fertility of interspecific crosses lies between 30 and 75% for parental and 33.3–66.6% for F_1. The proportion of fertile interspecific crosses compared to fertile intraspecific controls is found to be significantly lower (Mann–Whitney, $0.019 < P < 0.038$) with one possible exception, i.e. when *A. tibetiana* females mate with *A. sinica* males (see Table 2). This indicates that, under laboratory conditions, post-mating isolating barriers to gene flow are not so strong between *A. tibetiana* and Eastern Old World bisexuals and especially during F_1, while in most cases there is obvious hybrid breakdown in F_2 and F_3 generations (see Table 2).

Allozyme analyses

Twenty enzyme-coding loci were successfully scored. However, difficulties (pronounced satellite banding) were encountered in the scoring of two loci, namely PGM and PEP-1. Fixed allelic differences were observed: in three enzyme systems (EST-4, LDH, LAP-3) between the FRA and TIB populations, in one (EST-1) between TIB and URM while between TIB and YIM there were none. Allele frequencies, observed and expected heterozygosities and probability values for conformity to H-W equilibrium are shown in Table 4.

Several tests for deviations from H-W expectations were performed in each population. After Bonferroni correction, most of the departures were seen to be associated with the Tibet sample (MDH-2, IDH-2, ME, PEP-1, IDH-1, GOT-1). They were all highly significant with probability values ranging from $<10^{-5}$ to 0.0095. In the YIM population only the genotype frequencies of the ME locus deviated significantly

Table 4. Allele frequencies and genetic variability estimates in five populations

Locus-Allele	FRA	TIB	YIM	VC	URM
EST-D 100	1.000	1.000	0.865	1.000	0.981
EST-D 150y	0.000	0.000	0.135	0.000	0.000
EST-D 90u	0.000	0.000	0.000	0.000	0.013
EST-D 80u	0.000	0.000	0.000	0.000	0.006
Ho			0.146		0.039
He			0.237		0.038
P			0.025		0.999
EST-1 100	1.000	1.000	1.000	1.000	0.000
EST-1 110u	0.000	0.000	0.000	0.000	1.000
EST-4 100	1.000	0.000	0.000	1.000	0.000
EST-4 110	0.000	1.000	1.000	0.000	1.000
6-PGDH 100	0.500	0.000	0.000	0.375	0.000
6-PGDH 150	0.500	0.000	0.000	0.625	0.000
6-PGDH 25	0.000	0.698	0.844	0.000	0.772
6-PGDH 75	0.000	0.302	0.156	0.000	0.137
6-PGDH 20u	0.000	0.000	0.000	0.000	0.091
Ho	0.458	0.396	0.271	0.458	0.389
He	0.505	0.426	0.266	0.474	0.378
P	0.568	0.737	0.999	0.999	0.116
MDH-1 100	1.000	1.000	1.000	1.000	0.968
MDH-1 110u	0.000	0.000	0.000	0.000	0.006
MDH-1 90u	0.000	0.000	0.000	0.000	0.026
Ho					0.064
He					0.063
P					0.999
MDH-2 100	1.000	0.875	1.000	1.000	0.039
MDH-2 125	0.000	0.125	0.000	0.000	0.013
MDH-2 90u	0.000	0.000	0.000	0.000	0.948
Ho		0.000			0.090
He		0.221			0.100
P		$<10^{-5}$*			0.103
LDH 100	1.000	0.000	0.000	1.000	0.000
LDH 85	0.000	1.000	1.000	0.000	1.000
IDH-1 50	0.125	0.000	0.000	0.135	0.058
IDH-1 100	0.875	0.625	0.323	0.865	0.006
IDH-1 150	0.000	0.375	0.677	0.000	0.930
IDH-1 80u	0.000	0.000	0.000	0.000	0.006
Ho	0.125	0.250	0.313	0.146	0.116
He	0.221	0.474	0.442	0.237	0.135
P	0.010	0.001*	0.049	0.027	0.005
IDH-2 100	0.917	0.000	0.927	0.969	0.844
IDH-2 135	0.083	0.000	0.000	0.031	0.020
IDH-2 65	0.000	0.635	0.000	0.000	0.026
IDH-2 85	0.000	0.365	0.052	0.000	0.065
IDH-2 115	0.000	0.000	0.021	0.000	0.045
Ho	0.125	0.146	0.146	0.021	0.259
He	0.154	0.468	0.139	0.061	0.281
P	0.267	$<10^{-5}$*	0.999	0.033	0.193
GOT-1 100	1.000	0.021	0.031	1.000	0.090
GOT-1 125	0.000	0.979	0.969	0.000	0.910
Ho		0.000	0.063		0.103
He		0.041	0.061		0.166
P		0.009*	0.999		0.011
GOT-2 100	0.969	0.000	0.000	1.000	0.967
GOT-2 85	0.031	1.000	0.021	0.000	0.007
GOT-2 75	0.000	0.000	0.979	0.000	0.026
Ho	0.063		0.042		0.039
He	0.061		0.041		0.063
P	0.999		0.999		0.063

Table 4. *Continued*

Locus-Allele	FRA	TIB	YIM	VC	URM
LAP-2 100	1.000	1.000	1.000	1.000	0.007
LAP-2 90u	0.000	0.000	0.000	0.000	0.993
Ho					0.013
He					0.013
P					0.999
LAP-3 100	1.000	0.000	0.000	1.000	0.941
LAP-3 90	0.000	1.000	1.000	0.000	0.059
Ho					0.090
He					0.110
P					0.222
PGM 100	0.854	0.000	0.000	0.875	0.000
PGM 90	0.135	0.000	0.000	0.125	0.000
PGM 80	0.010	0.000	0.000	0.000	0.539
PGM 60	0.000	0.906	0.990	0.000	0.461
PGM 50	0.000	0.094	0.010	0.000	0.000
Ho	0.125	0.188	0.021	0.125	0.298
He	0.255	0.172	0.021	0.221	0.500
P	0.001*	0.999	0.999	0.013	0.0001*
PGI 65	0.271	0.000	0.000	0.354	0.013
PGI 100	0.729	0.000	0.000	0.646	0.000
PGI 15	0.000	0.823	1.000	0.000	0.910
PGI 50	0.000	0.177	0.000	0.000	0.071
PGI 10u	0.000	0.000	0.000	0.000	0.006
Ho	0.458	0.313		0.542	0.181
He	0.399	0.295		0.462	0.169
P	0.457	0.999		0.338	0.994
PEP-1 65	0.281	0.000	0.000	0.375	0.000
PEP-1 100	0.719	0.000	0.000	0.625	0.993
PEP-1 85	0.000	0.260	0.594	0.000	0.007
PEP-1 115	0.000	0.740	0.406	0.000	0.000
Ho	0.229	0.188	0.354	0.250	0.013
He	0.409	0.389	0.488	0.474	0.013
P	0.002*	0.0007*	0.075	0.001*	0.999
PEP-4 100	1.000	1.000	0.990	1.000	1.000
PEP-4 115y	0.000	0.000	0.010	0.000	0.000
Ho			0.021		
He			0.021		
P			0.999		
ME 100	0.979	0.000	0.000	1.000	0.000
ME 90f	0.021	0.000	0.000	0.000	0.000
ME 65	0.000	0.833	0.813	0.000	1.000
ME 85	0.000	0.167	0.188	0.000	0.000
Ho	0.000	0.000	0.000		
He	0.041	0.281	0.308		
P	0.011	$<10^{-5}$*	$<10^{-5}$*		
SOD 100	1.000	1.000	1.000	1.000	1.000
AMY 100	1.000	1.000	1.000	1.000	1.000
N	48	48	48	48	77
MNA	1.5 (0.10)	1.5 (0.10)	1.5 (0.10)	1.3 (0.10)	2.3 (0.10)
%P	30	40	30	25	40
Mean Ho	0.079 (0.032)	0.074 (0.028)	0.069 (0.026)	0.077 (0.036)	0.085 (0.025)
Mean He	0.102 (0.037)	0.138 (0.041)	0.101 (0.035)	0.096 (0.039)	0.101 (0.031)

Ho, observed heterozygosity; He, expected heterozygosity (unbiased estimate, Nei, 1978); %P, percentage of polymorphic loci (0.95 criterion); MNA, mean number of alleles per locus; *P*, probability of conformity to H-W expectations (exact test); *N*, sample size; f, allele private to FRA; y, allele private to YIM; u, allele private to URM. Standard errors in parentheses.
*Significant H-W deviations (Bonferroni correction).

Table 5. Single-locus F_{IS} values differing significantly from zero

Locus/Population	FRA	TIB	YIM	VC	URM
MDH-2		1.000***			
IDH-1		0.467**			
IDH-2		0.685***		0.656***	
GOT-1		1.000***			0.371**
GOT-2					0.383***
PGM	0.504***				0.398***
PEP-1		0.513***		0.467**	
ME	1.000***	1.000***	1.000***		

**$P < 0.01$, ***$P < 0.001$.

Table 6. Summary of *F*-statistics for polymorphic loci in five populations

Locus/Index	F_{IS}	F_{IT}	F_{ST}
EST-D	0.304	0.373	0.100***
EST-1	1.000	1.000	1.000***
EST-4	1.000	1.000	1.000***
6-PGDH	0.031	0.471	0.454***
MDH-1	–0.018	–0.002	0.015 NS
MDH-2	0.620	0.950	0.870***
LDH	1.000	1.000	1.000***
IDH-1	0.359	0.711	0.549***
IDH-2	0.332	0.679	0.519***
GOT-1	0.382	0.930	0.886***
GOT-2	0.186	0.955	0.945***
LAP-2	0.002	0.992	0.992***
LAP-3	0.183	0.955	0.945***
PGM	0.365	0.780	0.653***
PGI	–0.125	0.571	0.619***
PEP-1	0.416	0.746	0.565***
PEP-4	–0.001	$<10^{-3}$	0.001 NS
ME	1.000	1.000	0.824***
Mean	0.277	0.833	0.768***
Jacknife			
Average	0.270	0.832	0.768
SD	0.092	0.048	0.053
Bootstrap (95%CI)			
Upper	0.456	0.921	0.872
Lower	0.119	0.735	0.668

***$P < 0.001$; NS, not significant ($P > 0.05$); SD, standard deviation; CI, confidence intervals.

($P < 10^{-5}$). The same was true of URM at PGM ($P = 0.0001$). For the FRA and VC populations departures were observed for the PGM ($P = 0.0015$) and PEP-1 ($P = 0.0026$), and PEP-1 ($P = 0.0017$) loci, respectively. Heterozygote deficiency was the dominant type of deviation across samples.

The allele frequencies in 13 out of 20 loci (65%) were significantly different between FRA and TIB as well as between TIB and URM, with a combined probability of $<10^{-5}$. Similarly, for the FRA–YIM comparison the allele frequencies were significantly different for 13 loci (combined $P < 10^{-5}$) while a percentage of 70% of loci (14 out of 20) differed between FRA and URM (combined $P < 10^{-5}$). For the pair TIB–YIM, significant differences in allele frequencies occurred in 7 loci out of 20 (35%). After Bonferroni adjustment the highest significant probability reached only 0.0004 with the combined probability being again $<10^{-5}$. Mean observed heterozygosities per locus (Ho) ranged from 0.069 (YIM) to 0.085 (URM). For the Tibet population, mean Ho was 0.074, not substantially different from that of the YIM sample (Wilcoxon test, $P = 0.953$). None of the remaining pairwise comparisons between populations showed any significance.

The URM population displayed the highest value for the mean number of alleles per locus (see Table 4) while the percentage of polymorphic loci was relatively invariable. The TIB and URM populations displayed the highest value (40%), though not significantly so. Eleven private alleles were identified across samples with frequencies ranging from 0.006 for MDH-1 110, IDH-1 80, PGI 10 (URM) to 1.000 for EST-1 110 (URM). The FRA population displayed one whereas TIB and YIM showed none and two, respectively (see Table 4).

Wright's *F*-statistics for population substructuring are presented in Tables 5 and 6. F_{ST} values were tested for significance according to Workman & Niswander (1970). The significance of F_{IS} values was determined by the formula of Li & Horvitz (1953). The sequencial Bonferroni correction for multiple tests was employed. Also shown are variance estimates (after jacknifing over loci) and 95% confidence intervals (bootstrap, 5000 replications). With the exception of the PEP-4 and MDH-1 loci, all the remaining F_{ST} values were highly significant ($P < 10^{-5}$). The overall mean F_{ST} value was 0.768 meaning that, of the total genetic variation, 76.8% is attributable to differences among

populations. The comparable value for the pair TIB–YIM was 44.1% (data not presented). The fixation index F_{IS} (Table 5) may also be interpreted as describing departures from the expected H-W genotype frequencies within local populations (Nei, 1973). Positive values indicate heterozygote deficiencies. In addition, estimates of gene flow are commonly obtained by the F_{ST} values (Wright, 1951). Roughly speaking, the average exchange of 1 individual per generation ($Nm \sim 1$) between populations is marginally sufficient in theory to prevent differentiation by genetic drift alone (Allendorf, 1983). The value $Nm = 1$ corresponds to mean $F_{ST} = 0.20$. For the TIB and YIM populations, $Nm = 0.31$, representing less than 1 individual per generation.

Nei's (1978) matrix of genetic distances is shown in Table 7 with its graphical representation in Figure 1. Bootstrap values are given along branches. Large genetic distances are observed between FRA-TIB and FRA-YIM populations ($D = 0.730$ and 0.689, respectively). The genetic distance value for the comparison Tibet vs. Yimeng was 0.114 while that for Tibet vs. Urmia was 0.475. However, caution is needed in the interpretation of the results since a number of loci produced tied trees.

Table 7. Matrix of Nei's pairwise unbiased (1978) genetic distance for five populations

Population	FRA	TIB	YIM	VC	URM
FRA	–				
TIB	0.7304	–			
YIM	0.6892	0.1147	–		
VC	0.0008	0.7381	0.6875	–	
URM	0.7510	0.4758	0.3964	0.7571	–

RAPD ANALYSES

Gel profiles between populations and the resulting dendrogram are shown in Figure 2. *A. persimilis* is well differentiated from the Old World populations. Within the Old World, parthenogens are grouped together and close to their possible bisexual ancestor (*A. urmiana*, Abreu-Grobois, 1987). The position of *A. franciscana* within the Old World cluster appears to be somewhat problematic, as judged from our current knowledge of *Artemia* phylogeny based on allozymes (Abreu-Grobois, 1983, 1987; Pilla, 1992). RAPD markers screen different portions of the genome, a fact that, in some cases, may give rise to discordant patterns. Finally, the branching pattern for the two

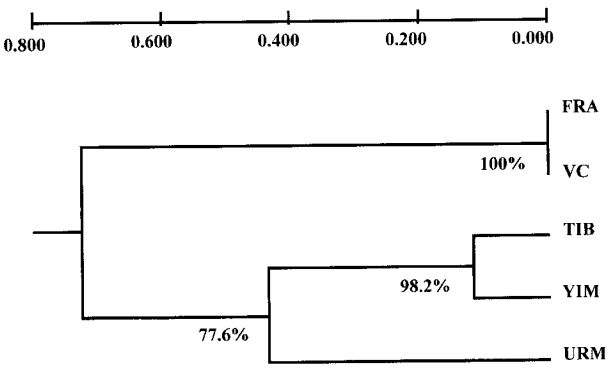

Figure 1. UPGMA dendrogram of Nei's unbiased (1978) genetic distance, showing bootstrap values (%) of node support out of 5000 permutations.

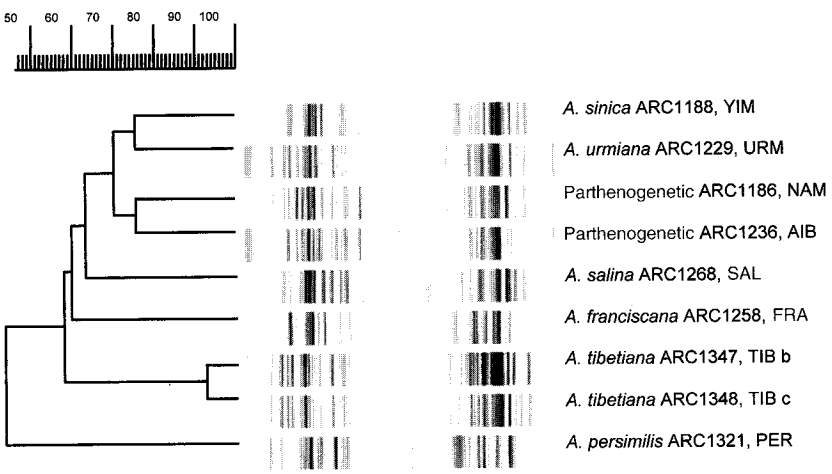

Figure 2. UPGMA dendrogram obtained from the RAPD gel profiles.

Tibetan populations shows that they have a high degree of similarity to each other and a marked divergence from the remainder of the Old World populations.

DISCUSSION

The characterization of species and populations has been a focus of interest during the last fifty years. Many of the factors thought to be responsible for genetic differentiation and speciation in other species (Barigozzi, 1982) are observable in *Artemia*: ecological isolation, formation of clines in heterochromatin content, poly, hetero- and aneuploidy, parthenogenesis and pre- or post-mating reproductive isolation. One of the molecular tools early employed to answer a wide range of questions regarding speciation processes has been protein electrophoresis (Ayala, 1976). Considering the pronounced tendency of *Artemia* populations for the development of local adaptations, electrophoretic surveys have been highly informative for the genus.

In this study, a battery of 20 molecular markers was used to investigate a number of genetic variability parameters for five *Artemia* populations and thereby evaluate the taxonomical status of the newly discovered Tibetan population. Considering the underestimation of genetic differences due to limitations of protein electrophoresis, a proportion of *at least* 2/3 of genetic loci differ in allele frequencies between FRA and TIB *Artemia*. For the same pair of populations the mean genetic distance estimate (D = 0.730) falls well within the range (0.098–1.501) of mean congeneric comparisons for bisexual *Artemia* (Beardmore *et al.*, 1995). It is interesting though that the FRA-TIB mean D-value is substantially lower from typical Old vs. New World species comparisons (on average >1 electrophoretically detectable allelic substitutions per locus) despite the large standard errors involved in those latter estimates. There is no doubt that considerable genetic differentiation exists between the FRA and TIB populations and as a consequence complete reproductive isolation has developed as gauged by cross-breeding/fertility tests. In addition, FRA and TIB individuals are morphologically distinct (our unpubl. data), evidence that organismal evolution has proceeded in parallel with molecular divergence. The genetic differences accumulated during, and subsequent to, speciation have made an impact on the majority of the genome. By the time the two lineages have effectively diverged, they have changed considerably in gene content and almost completely in allele frequency distribution.

A substantial amount of differentiation is also revealed when the TIB and URM populations are compared. Allele frequency differences occur at 65% of loci. The URM population displayed the highest number of private alleles (see Table 4) and appears to be well divergent (see dendrogram in Fig. 1) from the Eastern Asia cluster (TIB and YIM) from which it is separated by a satisfactorily high (77.6%) bootstrap value of node support. Genetic distance (D) values for individual comparisons (TIB vs. URM and YIM vs. URM equal 0.475 and 0.396, respectively) are typically congeneric (Beardmore *et al.*, 1995).

A quite different situation is revealed for the comparison of Tibet and Yimeng populations. Interestingly, these two populations show no fixed allelic differences, although 35% of their loci varied significantly in allele frequencies. Deviations from H-W proportions are prevalent in Tibet (six overall) compared to all other populations whereas the Yimeng sample shows the least (only one for the ME locus). The high occurrence of deviations in Tibet *Artemia* could be due to a variety of factors (e.g. Wahlund effect, selection against heterozygotes, miscoring, null alleles, etc.) but it may also indicate some degree of subdivision. Lagkor Co is not a large body of water, making the possibility of local inbreeding seem remote. Nevertheless, only systematic and seasonal sampling from a number of sites within the lake can safely evaluate this hypothesis. Mean observed heterozygosities between TIB and YIM are of the same magnitude (0.074 and 0.069, respectively) and there is no considerable variation in the mean number of alleles per locus or the percentage of polymorphic loci. However, a number of loci (EST-D, MDH-2, IDH-1, IDH-2, GOT-2, PGI and PEP-1) can, with a high degree of confidence, be considered as diagnostic between TIB and YIM. It is reasonable to suppose that this fact may be reflected by the estimated value of gene flow (Nm = 0.31). Additionally, 44.1% of the genetic variation shared by Tibet and Yimeng *Artemia* is due to interpopulational differences, a value consistent with separate species status (Pilla & Beardmore, 1994). In contrast, the mean F_{ST} value for *A. sinica* populations is only 6% (Beardmore *et al.*, 1995). Moreover, the relative strength of the node leading to TIB and YIM (see Fig. 1) is supported by an impressive 98.2% of permutated data sets, presumably as a result of marked differences in allele frequencies at 1/3 of loci.

It seems that genetic differentiation is in an initial stage and the combined forces of natural selection and genetic drift have started to produce divergent gene pools. Mean genetic distance values similar to the one observed between Tibet and Yimeng (D = 0.114) are normally associated with conspecific populations of bisexual *Artemia* (mean D , 0.109, Abreu-Grobois, 1987). Nonetheless, there are many well-documented cases, e.g. Hawaiian *Drosophila*, where differences between species are surprisingly limited (Carson *et al.*, 1975; Templeton, 1980; Avise, 1994) and, for that

matter, *Artemia* is not an exception. The genetic distance of *A. franciscana* vs. Mono Lake population (considered by many as a separate species, *A. monica*) amounts to only ≈ 10% ($D = 0.098$) of the genome, due solely to strong ecological isolation (Beardmore & Abreu-Grobois, 1983). Speciation involving a very small number of genes has been reported elsewhere (Tauber & Tauber, 1977; Tauber *et al.*, 1977). There is a good possibility that this is also the case for Tibet and Yimeng *Artemia*. Our data on morphological markers (to be published) have revealed differences between respective individuals, of the same order of magnitude as those observed when Tibetan and shrimps from other *Artemia* species are compared. In addition, the proportion of fertile homotypic crosses was in most cases significantly higher than with interspecific ones (TIB–YIM). Post-mating isolating barriers may rely on a few regulatory genes, not assayable by allozyme electrophoresis. Given an appreciable genetic divergence, superimposition of reproductive isolation does not by itself demand many additional gene differences (Dobzhansky, 1976).

With regard to the bisexual species identified by allozymes, the specific status of *A. tibetiana* is also confirmed by AFLP (Sun *et al.*, 1999) and RAPD markers (in this study). *A. persimilis* and *A. tibetiana* are divergent from all other species (Fig. 2). Ideally, we would expect *A. franciscana* to be on the same branch as *A. persimilis*, and well separated from the Old World cluster. However, this is not the case and may be due to the fact that certain genetic events (e.g. chromosome pair duplication, divergence in the number of nuclear chromocentres) in *A. persimilis* postdate its separation from *A. franciscana* (Beardmore & Abreu-Grobois, 1983). It is likely that such events have greatly affected the RAPD-derived phylogeny shown in Figure 2. In general, the genetic differences scored by RAPD markers are closely parallel to those obtained by protein electrophoresis and/or morphological traits (our unpubl. data). DNA assays like those above could significantly contribute to the investigation of evolutionary problems both in the intraspecific and interspecific levels.

Conversions of genetic distance measures to estimates of time since recent shared ancestry are controversial and not always legitimate (Abreu-Grobois & Beardmore, 1982). They have been also extremely variable depending on whether the source DNA was nuclear or mitochondrial (Perez *et al.*, 1994). Nevertheless, such methods have been used with some success in conjunction with available geological records (Sarich & Cronin, 1977; Vawter *et al.*, 1980). The most widely used formula for the conversion of measures of genetic relatedness is due to Nei (1975). It estimates the divergence time (t) between two lineages from the genetic distance value (D) as $t = D/2\alpha$, where α is the rate of electrophoretically detectable mutations with a suggested value of 10^{-7} per year. According to our data, we obtain an estimated time since divergence of 3.65×10^6 years for the FRA–TIB separation, 570 thousand years for TIB–YIM and 2.38×10^6 years for TIB–URM. Avise (1994) and Ayala (1999) have reviewed the factors likely to cause over- or under-estimation of such measures, like generation time, population size, etc. However, it would be interesting to look particularly at the geomorphological changes in the Tibetan plateau since the last glaciation period in search of a plausible evolutionary scenario.

The genetic differences between Tibet and Yimeng *Artemia* suggest a recent separation. Based on the same allozymic criteria as this study, genetic distance values of the same magnitude as TIB–YIM are typical for conspecific populations of the most highly structured species, *A. franciscana* (mean D , 0.126, Beardmore *et al.*, 1995). The likelihood of extensive geographical differentiation cannot be completely ruled out, especially with the limited number of populations investigated here, a fact that can lead to a fallible taxonomy. Certainly, *Artemia* samples from other lakes of the Tibetan plateau need to be assayed. However, our data firmly suggest a geographical mode of speciation, where much of the divergence is the result of adaptation to different environments, but other factors such as genetic drift may play a role as well. Presumably, the presence of a few or no differentiating electromorphs between closely related species indicates that insufficient time has elapsed for the accumulation of greater *de novo* mutational differences in these assays (Avise, 1994).

Many instances indicate that not all speciation events involve the same amount of genetic change (Dobzhansky, 1976), an illustration of the tremendous heterogeneity of the biological world. The genus *Artemia* is rather depauperate but, admittedly, it shows no shortage of speciation mechanisms or genic variability available for conversion to between-species genetic differences. In the light of our data, *Artemia* from Tibet strongly qualifies as a separate species with the proposed name 'Artemia tibetiana'.

The results of the present study provide genetic evidence for the specific status of *Artemia* from Tibet. Laboratory reproductive isolation from New and Old World bisexual species has been demonstrated as well as genetic divergence by allozyme and RAPD markers. Our findings are further supported by biometric, cytogenetic (Abatzopoulos *et al.*, 1998) and AFLP data (Sun *et al.*, 1999). The mechanisms involved in the speciation process in the *Artemia* genus are manifold. We emphasize the need of multidisciplinary approaches for species designations in the genus. Individual and/or collective treatment of those different

data sets could greatly contribute to a better understanding of the evolutionary history of *Artemia* in the future. In conjunction with available geological data, it could also enable us to reconstruct phylogenies with increased confidence.

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